Binding, Surface Mobility, Internalization, and Degradation of Rhodamine-Labeled α_2-Macroglobulin†

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ABSTRACT: We have used quantitative fluorescence methods to examine the fate of rhodamine-labeled α_2-macroglobulin (R-α_2M) after binding to cell-surface receptors on NRK and Swiss 3T3 cells. From measurements of fluorescence intensities in NRK cells fixed after incubation with R-α_2M, we found that uptake was saturable and that half-maximal uptake occurred at 130 nM R-α_2M. Fluorescence measurements on cell extracts of NRK and Swiss 3T3 cells also showed a half-maximal uptake of R-α_2M near 130 nM. We estimate that NRK cells can take up 10^6 molecules of R-α_2M per hour via receptor-mediated endocytosis. The mobility of α_2-macroglobulin receptors on the surface of Swiss 3T3 cells was measured by using fluorescence photobleaching recovery. The two-dimensional effective diffusion coefficient of R-α_2M receptors was approximately 8×10^{-10} cm^2 s^{-1}, a value close to that previously obtained for insulin and epidermal growth factor receptors. Degradation of R-α^2M by the cells was followed by using the loss of fluorescence from the 185 000-dalton band in sodium dodecyl sulfate–polyacrylamide gels. Rhodamine fluorescence was detected in the gels by using a microscope fluorescence spectrophotometer. NRK cells degraded α_2M to low molecular weight fragments with a $t_{1/2}$ of 15 min. Swiss 3T3 cells degraded about 75% of the α_2M with a $t_{1/2}$ of 1 h. The remaining 25% remained as the intact 185 000-dalton peptide after 24 h. No significant accumulation of large breakdown products was observed in Swiss 3T3 or NRK cells.

Concentrative receptor-mediated endocytosis is the process used by cells for the rapid, selective uptake of molecules bound to cell-surface receptors (Silverstein et al., 1977; Goldstein et al., 1979; Pastan & Willingham, 1981; Maxfield et al., 1979c). In fibroblasts and other cell types, it has now been shown that several proteins and hormones, including insulin (Schlessinger et al., 1978b; Maxfield et al., 1978), epidermal growth factor (EGF) (Schlessinger et al., 1978b; Maxfield et al., 1978; Gorden et al., 1978; Haigler et al., 1978, 1979), low-density lipoproteins (Goldstein et al., 1979; Anderson et al., 1976), 3,3′,5-triiodo-L-thyronine (T_3) (Cheng et al., 1980), α_2-macroglobulin (α_2 M) (Maxfield et al., 1978, 1979c; Van Leuven et al., 1979; Kaplan & Nielsen, 1979), and several others [reviewed in Goldstein et al. (1979), Pastan & Willingham (1981), and Maxfield et al. (1979c)] enter cells by this process. Several features of this process have been elucidated. Clustering of occupied receptors in coated pits precedes the endocytosis of α_2M, insulin, EGF, and low-density lipoproteins by cultured fibroblasts (Maxfield et al., 1979c; Anderson et al., 1976; Willingham et al., 1979). The clearance of occupied receptors from the cell surface occurs with a half-time of less than 10 min at 37 °C (Pastan & Willingham, 1981; Haigler et al., 1979; Willingham & Pastan, 1980). The clustering of occupied α_2M receptors can be inhibited by many small molecules. These substances all are inhibitors of one form of cellular transglutaminase activity, implying that transglutaminase or a transglutaminase-like enzyme may be involved in some forms of receptor clustering (Maxfield et al., 1979a; Davies et al., 1980).

We have found that fluorescently labeled α_2-macroglobulin is a very useful ligand for studying receptor-mediated endocytosis. α_2M enters cultured fibroblasts by the same pathway as insulin or EGF (Maxfield et al., 1978), but its large size allows more extensive labeling than is possible for the hormones. Rhodamine-labeled α_2M (R-α_2M) has been used, for example, in studies in which inhibitors of clustering were identified because the bright fluorescence of R-α_2M could be seen easily, even when bound to diffusely distributed receptors.

The uptake of [^{125}I]α_2M has been studied in human fibroblasts (Van Leuven et al., 1979), rabbit macrophages (Kaplan & Nielsen, 1979), and in cultured rat fibroblasts (Dickson et al., 1981). We have shown qualitatively that R-α_2M uptake by cultured mouse fibroblasts is a saturable process (Maxfield et al., 1978). In this paper, we describe quantitative microscopic spectrofluorometric studies of the uptake and degradation of R-α_2M by Swiss 3T3 and NRK cells. We show that the concentration dependence of the uptake can be measured in situ or in cell extracts. The variability of the uptake among cells can be assayed by measuring fluorescence intensities of individual cells. We have also measured the mobility of R-α_2M bound to its cell-surface receptor using fluorescence photobleaching recovery.

Materials and Methods

Materials. α_2-Macroglobulin was prepared from whole human plasma by a slight modification (Willingham et al., 1979) of the procedure of Wickerhauser & Hao (1972). Rhodamine-labeled α_2M (R-α_2M) was prepared as previously described (Maxfield et al., 1978). The absorption maximum for R-α_2M was at 553 nm, and the ratio of OD_{553}/OD_{280} was 0.5. In the fluorescence spectrum, the excitation peak was seen at 553 nm, and the emission peak was at 575 nm. Protein concentrations were determined by the method of Lowry et al. (1951) as modified by McDonald & Chen (1965).

Methylamine hydrochloride, bacitracin, and all sugars used for binding inhibition were from Sigma. Bovine trypsin and soybean trypsin inhibitor were from Boeringher Mannheim. Dansylcadaverine was from Fluka.

Cells. Swiss 3T3-4 mouse fibroblastic cells were obtained and propagated as previously described (Willingham & Pastan, 1978). A clone of NRK cells with a fibroblastic morphology (NRK-2T) was obtained from Dr. G. Todaro and grown in

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Dulbecco–Vogt-modified Eagle's medium with 10% heated calf serum at 37 °C.

Fluorescence in Intact Cells. NRK cells were plated at 3 × 10^5 cells per 35-mm dish and incubated for 2 days. The cells were then rinsed gently twice with serum-free Dulbecco–Vogt-modified Eagle's medium (SFM) at 37 °C. For concentration dependence studies, various concentrations of R-α_2M were added to the cells in 1 mL of SFM, and the cells were incubated at 37 °C for 30 min. At the end of the incubation, the cells were rinsed 4 times with warm SFM and once in Dulbecco's phosphate-buffered saline (PBS). The cells were then fixed for 5 min in 2% formaldehyde in PBS and rinsed in PBS. Mountng fluid (DIFCO) was put on the fixed cells, and a no. 1 cover slip was placed on top.

For the time course of degradation measurements, the cells were incubated with R-α_2M, then rinsed 4 times with SFM, and incubated with 10% calf serum at 37 °C for various times. At the end of this incubation, the cells were rinsed twice with SFM and once with PBS and fixed as described above.

Fluorescence intensities were measured by using an EMI 9658 R photomultiplier mounted on a Zeiss standard microscope equipped for incident light fluorescence. Incident light from a 50-W mercury lamp was passed through an Instruments SA Model H10 monochromator with a spectral bandpass of 8 nm at 546 nm. An FI 546 exciter filter, FI 580 reflector, and LP 590 barrier filter were used. A 63× (N.A. 1.4) objective was used, and a circle of approximately 30-μM diameter was illuminated. Intensities were determined by measuring the current on the photomultiplier using a Keithley Model 480 picoammeter.

Measurements were made at 15–20 randomly selected sites on the dish after focusing near the top surface of the cells using low-level phase-contrast illumination. The average intensities measured on duplicate dishes were usually within 10%.

Fluorescence in Cell Extracts. NRK cells were grown and incubated as described above. Swiss 3T3 cells were plated at approximately half confluency and grown for 2 days in 10% calf serum. Incubations with R-α_2M were performed as described above. After the final rinse, the PBS was completely drawn off with a Pasteur pipet, and 150 μL of buffer containing 6% sodium dodecyl sulfate ($NaDodSO_4$, 20% glycerol, and 20 mM sodium phosphate (pH 6.8) was added to each dish. The dissolved cells were scraped with a rubber policeman and transferred to a test tube with a Pasteur pipet. Water (150 μL) was added to the dish, which was again scraped with the rubber policeman, and this wash was added to the test tube. 2-Mercaptoethanol (15 μL) was added to each tube, and the tubes were placed in a boiling water bath for 3 min.

The fluorescence intensity was measured by using the microscope spectrophotometer described in the preceding section. The sample (10 mL) was placed in one well of a cell-counting chamber (Scientific Products) according to the manufacturer's instructions, and a standard solution containing 2 μg/mL of R-α_2M in the same buffer was placed in the other well. The chambers have a thickness of 0.1 mm. A 16× dry objective (N.A. 0.4) was focused on the markings of the chamber, and the fluorescence intensity was measured. The intensity in the sample well was compared to the intensity of the standard solution to obtain the concentration of R-α_2M in the sample. Duplicate determinations were made on each sample and averaged. Values from duplicate dishes generally agreed within 10%. Concentrations of R-α_2M as low as 300 ng/mL (100 ng/dish) could be reproducibly measured by this technique. The low concentration, small volume, and high light scattering in the samples precluded the use of standard fluorescence measurements. The validity of the technique was tested by adding known amounts of R-α_2M to extracts from untreated cells.

Fluorescence Measurements in Polyacrylamide Gels. Samples were prepared as in the preceding section, and $NaDodSO_4$–polyacrylamide gel electrophoresis was performed by using a system described earlier (Wallach et al., 1978) with 3% stacking and 7% resolving polyacrylamide gels. No dye was added along with the fluorescent samples. Sample (20 μL) was loaded onto a gel lane approximately 3 mm wide. After electrophoresis, the gel was placed on a flat surface and covered with a clear polyethylene to prevent drying. The fluorescence intensities in the gel were measured immediately with the fluorescence microscope spectrophotometer with a 16× (N.A. 0.40) dry objective. The positions of the fluorescent bands were determined by reference to fixed points on the microscope stage. In order to determine concentrations, intensities were compared with lanes in the same gel which contained known amounts of R-α_2M added to extracts from untreated cells. The intensity was linear up to 700 ng of R-α_2M/lane, and as little as 3 ng of R-α_2M/lane could be reproducibly measured; 0.5 ng of R-α_2M/lane could be detected, but the errors at this concentration rose up to ±50%.

Fluorescence Photobleaching Recovery. Swiss 3T3 cells grown on coverslips were incubated with R-α_2M, and the lateral mobility of cell-surface receptors was determined by fluorescence photobleaching recovery (Schlessinger et al., 1977, 1978a). One group of cells was incubated in serum-free medium containing 120 μg/mL R-α_2M for 30 min at 23 °C. Other cultures were incubated in serum-free medium containing 20 mM methylamine or 2 mg/mL bacitracin for 30 min at 37 °C prior to incubation with 120 μg/mL R-α_2M for 30 min at room temperature in the same medium. A fourth group of cultures was incubated in serum-free medium containing 200 μg/mL R-α_2M on ice for 1 h. At the end of the incubations, the cells were rinsed 4 times with serum-free medium at room temperature, and the coverslips were inverted over hanging drop microscope slides. The medium in the slide chamber was buffered with 20 mM *N*-2-(hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.4). Methylamine or bacitracin was added to the medium in the slide if they had been present during the incubation. All fluorescence photobleaching recovery measurements were made at 23 °C.

Results

Concentration Dependence of Uptake. The concentration-dependent uptake of R-α_2M by NRK and Swiss 3T3 cells is shown in Figure 1. It should be emphasized that this uptake represents both binding and internalization (Maxfield et al., 1978; Willingham et al., 1979), since the cells were incubated with R-α_2M for 30 min at 37 °C. The uptake of R-α_2M by NRK cells was measured in three ways, as shown in Figure 1A: (1) the fluorescence intensity of fixed cells was measured directly on the dish; (2) the rhodamine fluorescence was measured in cell extracts; and (3) cell extracts were run on $NaDodSO_4$–polyacrylamide gels, and the rhodamine fluorescence in the gels was measured. All three methods show similar concentration-dependence curves for R-α_2M uptake. The lower values obtained with gels probably reflect partial degradation of the α_2M during a 30-min incubation (see below). From the fluorescence intensity in cell extracts, we estimate that at a concentration of 150 μg/mL each cell takes up about 4 × 10^5 molecules or R-α_2M in a 30-min incubation. Similar results were obtained with Swiss 3T3 cells (Figure 1B). The uptake of R-α_2M is half-maximal at about 100 μg/mL.

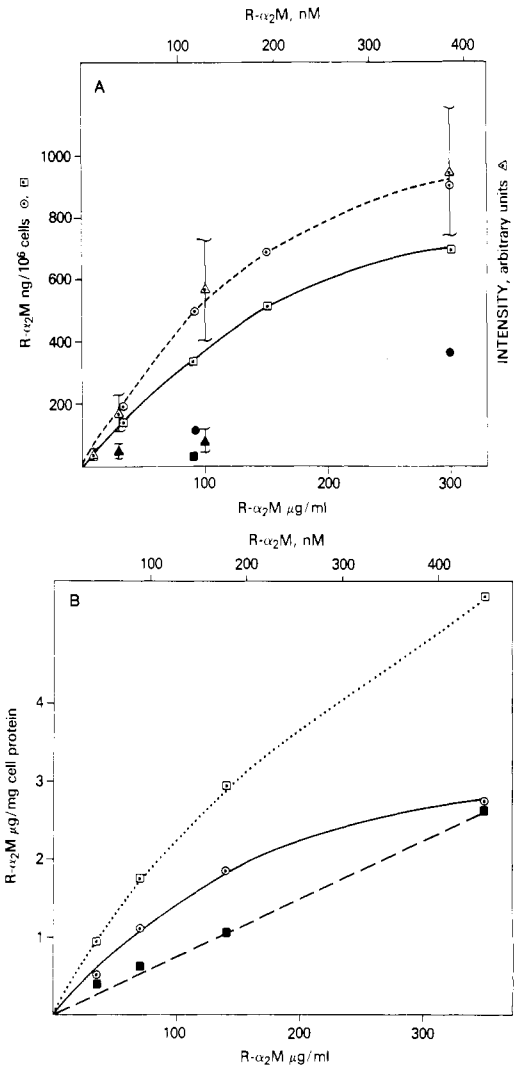

FIGURE 1: Concentration dependence of R-α_2M uptake. (A) NRK cells were incubated with various concentrations of R-α_2M for 30 min at 37 °C. The amount of R-α_2M associated with the cells was determined by measurements (△) on intact cells (○) in cell extracts or (□) by measuring the intensity of the 185 000-dalton band in $NaDodSO_4$–polyacrylamide gels as described under Materials and Methods. The solid symbols were the intensities measured on cells incubated in the presence of 3 mg/mL unlabeled α_2M. (B) Swiss 3T3 cells were incubated with α_2M for 30 min at 37 °C, and the cell-associated R-α_2M was determined by measuring the fluorescence in cell extracts as described under Materials and Methods. (□) Intensity in cell extracts; (■) intensity when the incubations were done in the presence of 3 mg/mL unlabeled α_2M; (○) fluorescence corrected for noncompetible binding.

The measurement of fluorescence intensities of individual NRK cells (Figure 1α) shows that there is considerable heterogeneity in the amount of R-α_2M taken up by individual cells. At 100 μg/mL R-α_2M, the standard deviation in the intensities was nearly one-third of the total binding. The 30-±m measuring diameter was chosen so that there was very little contribution to each intensity measurement from neighboring cells.

The binding of R-α_2M is not affected by treatment of the α_2M with trypsin to form protease–α_2M complexes. NRK cells incubated with R-α_2M or trypsin-treated R-α_2M at 30 μg/mL showed the same average fluorescence intensity (within 15%). Both the specific and nonspecific components of the binding were unaffected by trypsin treatment of the R-α_2M. In order to see if trypsin-treated α_2M was more efficient in blocking R-α_2M binding, we measured R-α_2M uptake in the presence of a small excess of α_2M or trypsin-treated α_2M.

Table I: Diffusion Coefficient of R-α_2M on Swiss 3T3 Cells at 23 °C

binding conditions	D (× 10^{-10} cm^2/s)	fractional recovery[a] (%)	no. of cells
on ice	7.2 ± 2.1	65 ± 16	14
methylamine	7.8 ± 3.5	53 ± 7	22
bacitracin, 2 mg/mL	9.6 ± 5.6	56 ± 11	13
30 min, 23 °C[b]	5.5 ± 8.0	37 ± 28	12

[a] The fractional recovery is the amount of fluorescence intensity recovered after the bleach. For proteins, this value is usually less than 100%; this has been taken as an indication that some of the molecules may be immobile (Schlessinger et al., 1977, 1978a). [b] Five of 12 cells showed less than 20% recovery. These cells were excluded from the calculation of D.

When the uptake of R-α_2M (90 μg/mL) was measured in the presence of 240 μg/mL α_2M or trypsin-treated α_2M, 28% or 25% of the uptake was blocked, respectively. If trypsin-treated α_2M were taken up preferentially, we would have expected to see greater uptake of trypsin-treated R-α_2M and more inhibition of R-α_2M uptake by trypsin-treated α_2M. Studies with [^{125}I]α_2M have also shown that the uptake of α_2M by NRK cells is not affected by trypsin treatment of the α_2M.

Periodate treatment (Spiro, 1966) or R-α_2M did not significantly affect binding to Swiss 3T3 cells, nor did a wide variety of sugars,[1] indicating that the carbohydrate moieties of α_2M are probably not involved in its binding to cells.

Receptor Mobility. Unoccupied α_2M receptors are diffusely distributed on the cell surface. Following occupancy by α_2M, these receptors rapidly cluster over coated regions (Maxfield et al., 1979c; Willingham et al., 1979). This redistribution implies that the receptors are mobile in the plasma membrane, and we have used fluorescence photobleaching recovery (Schlessinger et al., 1977, 1978a) to directly measure the effective diffusion coefficient of R-α_2M bound to its cell-surface receptor on Swiss 3T3 cells. In order to prevent clustering, the cells were labeled at 4 °C or in the presence of methylamine or bacitracin (Maxfield et al., 1979a,b). The results of these experiments are shown in Table I. The diffusion coefficient of the mobile component of receptor-bound R-α_2M is about 8×10^{-10} cm^2 s^{-1}. This value is similar to the effective diffusion coefficients for rhodamine-labeled insulin and EGF bound to their cell-surface receptors (Schlessinger et al., 1978a). When cells were incubated with R-α_2M at room temperature without methylamine or bacitracin, clusters could be seen, and the recovery of fluorescence following bleaching was low (Table I). In the presence of methylamine or bacitracin, no clusters could be seen for at least 60 min at 23 °C, and the recovery following bleaching remained high.

Degradation of R-α_2M. α_2M is internalized in endocytic vesicles (Willingham et al., 1979) following clustering over coated pits. We have recently shown these are a unique class of uncoated vesicles and named these "receptosomes" (Willingham & Pastan, 1980). We have used fluorescence methods to follow the fate of α_2M after internalization. The results obtained with two cultured cell lines, NRK and Swiss 3T3, were quite different and are shown in Figures 2 and 3.

In the experiment shown in Figure 2, NRK cells were incubated with R-α_2M for 15 min at 37 °C. The cells were then

[1] The sugars tested were D(+)-mannose, α-L(−)-fucose, D(+)-galactosamine, 3-*O*-methylglucose, phenyl β-D-galactoside, D-mannosamine, D-glucosamine 6-phosphate, *N*-acetyl-β-D-mannosamine, *N*-acetylneuraminic acid, methyl α-D-mannoside, α-D(+)-glucose, and D(+)-galactose. The sugars were added to SFM at a final concentration of 50 mM, and the cells were incubated with 50 μg/mL R-α_2M for 20 min at 37 °C.

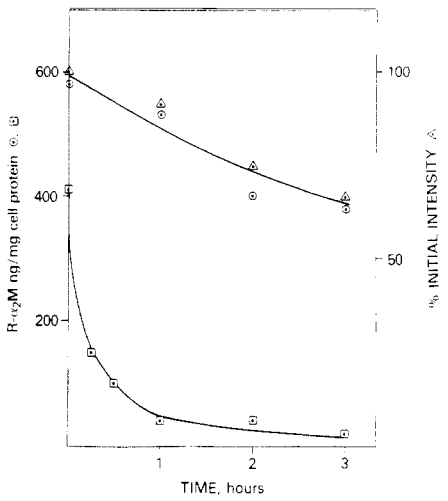

FIGURE 2: Degradation of R-α_2M by NRK cells. Confluent NRK cells were incubated with 120 μg/mL R-α_2M for 15 min at 37 °C. The cells were then rinsed and incubated in 10% calf serum for varying lengths of time. Total cell-associated fluorescence was measured (△) on intact cells or (○) in cell extracts. The amount of intact 185 000 molecular weight material was measured (□) in polyacrylamide gels.

rinsed extensively and incubated with medium containing 10% calf serum without R-α_2M for varying lengths of time. The total cell-associated fluorescence, as measured on fixed cells or in cell extracts, decayed slowly; more than 60% of the fluorescence remained after a 3-h incubation. However, when the amount of intact α_2M was followed by monitoring the intensity of the 185 000-dalton band in NaDodSO$_4$–polyacrylamide gels, we found that α_2M was degraded very rapidly by NRK cells. Roughly 60% of the 185 000-dalton band was lost during the first 15 min after removing R-α_2M from the medium, and more than 95% was lost during a 3-h incubation. Since the total cell fluorescence remained high, the loss of the 185 000-dalton band is due to degradation rather than release of α_2M into the medium. It appears that the fluorescent degradation products of R-α_2M are released slowly by NRK cells. Under similar conditions, ^{125}I-labeled EGF was degraded at approximately the same rate as that observed for R-α_2M (not shown).

In Swiss 3T3 cells (Figure 3), the degradation of R-α_2M is slower than that in NRK cells, and the degradation is biphasic. Roughly 25% of the R-α_2M initially bound to the cells remains as an intact 185 000-dalton peptide after 5 h. This 25% of the α_2M is not significantly degraded even after a 24-h incubation. The remaining 75% of the 185 000-dalton band is lost with a half-time of about 1 h. The loss of the 185 000-dalton peptide is predominantly due to degradation. When the medium from the incubation was trichloroacetic acid (Cl_3CCOOH) precipitated and run on an NaDodSO$_4$–polyacrylamide gel, we could not detect any significant release of specifically bound R-α_2M in the 185 000-dalton region (not shown). We estimate that up to 20% of the specifically bound R-α_2M could have been released without being detected. Again, the total cell-associated fluorescence decayed more slowly than the 185 000-dalton fluorescence, but since the degradation is slow, the difference is not as pronounced as in the NRK cells.

Most of the nonspecifically bound R-α_2M was rapidly released into the medium as the intact polypeptide at 37 °C. When Swiss 3T3 cells were incubated as described in Figure 3, 75–90% of the nonspecific (noncompetible) cell-associated fluorescence was removed by a 30-min incubation in 10% calf serum. Cl_3CCOOH precipitation of the medium followed by NaDodSO$_4$–polyacrylamide gel electrophoresis showed that

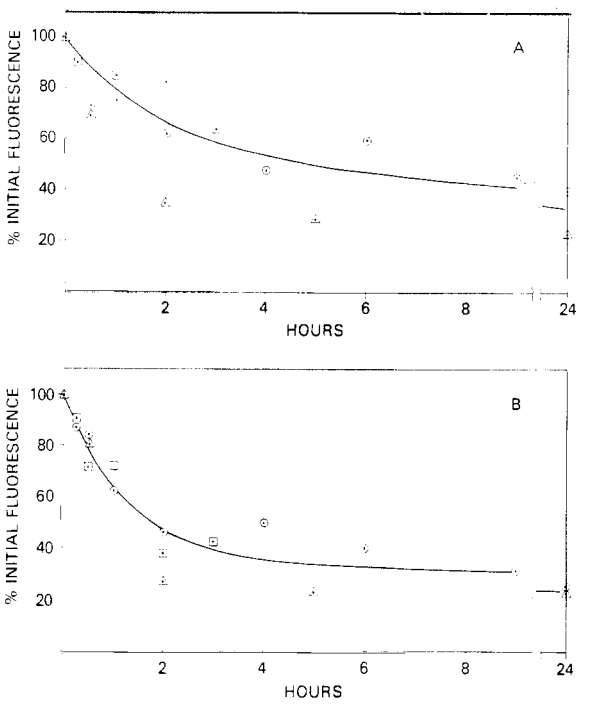

FIGURE 3: Degradation of R-α_2M by Swiss 3T3 cells. Swiss 3T3 cells were incubated with 120 μg/mL R-α_2M for 30 min at 37 °C. The cells were rinsed and incubated with 10% calf serum for various times. (A) Total cell fluorescence was measured in cell extracts. (B) The amount of 185 000 molecular weight material was measured in polyacrylamide gels. The different symbols represent results from separate experiments. The intensities are corrected for nonspecific binding.

the released material was intact R-α_2M.

Figure 4 shows fluorescence scans of NaDodSO$_4$–polyacrylamide gels after electrophoresis of (A) R-α_2M or (B–E) extracts from cells incubated with R-α_2M. As seen in Figure 4A, the major fluorescent component runs at an apparent molecular weight of 185 000, as expected for α_2M. There are small amounts of fluorescence in other bands, and a large percentage of the fluorescence runs near the front. The origin of the material at the front is not clear at this time. When samples were prepared for electrophoresis in the absence of reducing agents, the intensity of this band decreased by 70–80% (not shown). This indicates that some of the rhodamine is attached to the protein via a linkage which is susceptible to reduction by sulfhydryl. Since some fluorescence was detected at the front even in the absence of reducing agents, it also appears that some (10–20%) of the rhodamine may be noncovalently associated with the α_2M even after dialysis for several days with extensive buffer changes. Samples boiled for 20 min showed the same amount of fluorescence in the 185 000-dalton band as samples boiled for 3 min, indicating that the release of rhodamine by reducing agents and NaDodSO$_4$ is essentially complete in 3 min or less. We do not know the nature of the bonds which are susceptible to sulfhydryl reduction.

Figure 4B–E shows the fluorescence scans of samples obtained from solubilized cells after incubation with R-α_2M. When NRK cells were incubated with R-α_2M for 15 min, the fluorescence profile shown in Figure 4B was obtained. Subsequent incubation for 15 min in 10% calf serum without R-α_2M resulted in a substantial loss of intensity in the 185 000-dalton band, as shown in Figure 4C. Similar results are presented in Figure 4D,E for incubations with Swiss 3T3 cells. Although some new fluorescent bands appear in the R-α_2M samples after incubation with cells, no major high

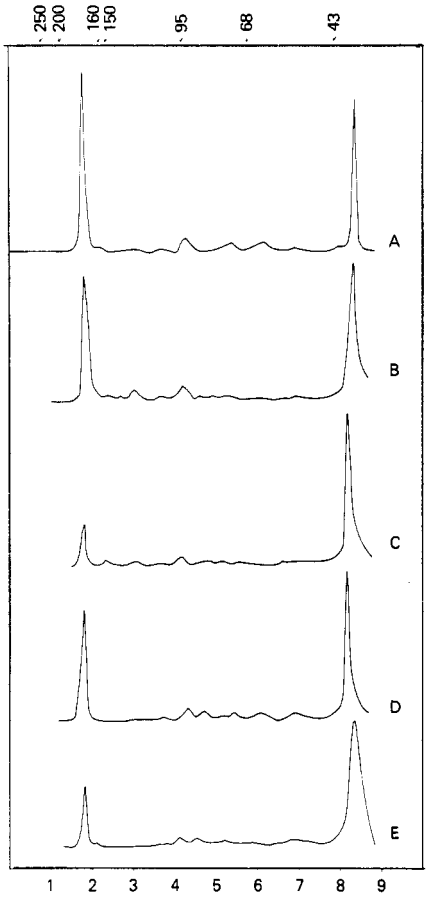

FIGURE 4: R-α_2M in polyacrylamide gels. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed, and the fluorescence intensity was analyzed as described under Materials and Methods. (A) R-α_2M; (B) cell extracts from NRK cells incubated with 120 μg/mL R-α_2M for 15 min at 37 °C; (C) NRK cells incubated as in (B), followed by 15 min in 10% calf serum; (D) Swiss 3T3 cells incubated with 120 μg/mL R-α_2M for 30 min at 37 °C; (E) Some as in (D), followed by 30 min in 10% calf serum. The molecular weight standards shown by arrows at the top of the figure are filamin, myosin, B,B′-RNA polymerase, phosphorylase *a*, bovine serum albumin, and ovalbumin.

molecular weight degradation products accumulate in the cells. For the data presented in Figures 1–3, we have used the peak height in the 185 000-dalton region as a measure of the intact R-α_2M in the cells. Since all samples were treated in the same way, the release of some low molecular weight fluorescent material should not affect the relative values obtained.

Discussion

Fluorescent hormones and proteins have recently been used in several studies of receptor-mediated uptake (Pastan & Willingham, 1981; Maxfield et al., 1979b,c; Schlessinger et al., 1977, 1978a,b; Willingham et al., 1979; Willingham & Pastan, 1980; LeCam et al., 1979), and these studies have provided valuable information about the mechanism and function of receptor-mediated endocytosis. In studies with fluorescent ligands, it is necessary to be sure that the fluorescent probes retain high affinity and specificity in their binding. It is also important to determine whether the fluorescent probe remains attached to the hormone or protein after interaction with the cells. In the past, affinity and specificity of binding have generally been assayed by testing the ability of the fluorescent ligand to block the binding of a radioactive ligand (Haigler et al., 1978; Cheng et al., 1979; Shechter et al., 1978). Whereas this method accurately measures binding to the receptor, the extent of nonspecific binding by the fluorescent ligand has been determined by observation of cells labeled in the presence of excess unlabeled ligand (Schlessinger et al., 1978b; Maxfield et al., 1978; Cheng et al., 1980). In this paper, we have described methods which can be used to quantitatively determine the amount and molecular weight of a fluorescent ligand which is associated with cells. We have used these techniques to obtain quantitative information about the binding, uptake, and degradation of R-α_2M by NRK and Swiss 3T3 cells.

Measurements of fluorescent intensities on cells in the culture dish provide a quantitative measure of the fluorescence which is seen through the microscope. One advantage of this method is that the uptake of the fluorescent ligand by single cells can be observed. This may be useful in studying uptake in mixed cell populations or in differentiating cell cultures. The standard deviations shown in Figure 1A are an indication of the differences observed in the uptake of R-α_2M by individual NRK cells. One disadvantage of this method is that the fluorescent intensities cannot be directly converted into concentrations. This difficulty is overcome by methods using cell extracts. In practice, the simplest way to measure the fluorescence intensity is by dissolving the cells and measuring the fluorescence in cell extracts, as described under Materials and Methods. The cell-counting chambers provide a convenient, reproducible sample chamber with a very small sample volume ($\sim$10 μL). By comparing the fluorescence intensities with known concentration standards, we could determine the amount of R-α_2M taken up by the cells (Figure 1). Using the fluorescence microscope to scan polyacrylamide gels provided very high sensitivity (0.5 ng of R-α_2M/lane) and allowed us to follow the degradation of R-α_2M by the cells (Figures 2–4).

The half-maximal uptake of R-α_2M by Swiss 3T3 or NRK cells is observed at about 100 μg/mL (130 nM). Van Leuven et al. (1979) have measured the uptake of [^{125}I]α_2M by human fibroblasts. They found that half-maximal uptake occurred at 300 nM for α_2M, but the half-maximal uptake for α_2M–trypsin complexes was observed at 37 nM. We were unable to detect any difference between the uptake of R-α_2M and R-α_2M–trypsin complexes. Furthermore, α_2M–trypsin complexes did not compete for R-α_2M binding any more efficiently than α_2M. A scan of R-α_2M run on a polyacrylamide gel (Figure 4) showed little of the M_r 85 000 band which is observed after α_2M interaction with proteases. There was no indication that the R-α_2M bound to cells contained more of this M_r 85 000 band, as would be expected if α_2M–protease complexes were selectively taken up. We do not yet understand the difference between our results and those of Van Leuven et al. (1979). It is possible that the cultured mouse and rat cell lines do not recognize the difference between human α_2M with or without protease.

Dickson et al. (1981) have investigated the uptake of [^{125}I]α_2M by NRK cells. At 37 °C, they found a concentration dependence of the uptake very similar to that shown in Figure 1. At 0 °C, they found two classes of binding sites. One class had $K_d \approx 0.4$ nM and 12 000 sites/cell; the second component had $K_d \approx 40$ nM and 4×10^5 sites/cell. No substantial differences were detected between the uptake or [^{125}I]α_2M and [^{125}I]α_2M–trypsin. At the concentrations of R-α_2M used in our study, we are predominantly looking at the low-affinity, high-capacity class of receptors.

The degradation of R-α_2M, as measured by the loss of fluorescence in the M_r 185 000 band in polyacrylamide gels, was strikingly different in NRK and Swiss 3T3 cells. In the NRK cells, degradation occurred rapidly after internalization. In the Swiss 3T3 cells, degradation was relatively slow (Figure

3), and 25% of the R-α_2M remained intact after 24 h. Electron microscopic studies (Willingham & Pastan, 1980) of α_2M uptake by Swiss 3T3 cells have shown that α_2M passes through a series of organelles after internalization. Within 5 min after internalization, α_2M is found in uncoated endocytic vesicles, which have been named receptosomes. After 30 min, most of the α_2M has been transferred to small lysosomes which are often found near the Golgi or GERL. After longer incubations, the α_2M is found in larger heterologous lysosomes which are visible as dark structures by phase-contrast microscopy (Willingham & Pastan, 1980). The rate of R-α_2M degradation observed in Swiss 3T3 cells (Figure 3) is not consistent with rapid degradation in the small lysosomes in the Golgi region. If degradation of α_2M occurs within these small lysosomes, the $t_{1/2}$ for this process must exceed 30 min. Alternatively, degradation of α_2M by Swiss 3T3 cells may occur primarily after transfer of α_2M to the larger heterologous lysosomes. We do not know the intracellular localization of the R-α_2M which is still intact after 24 h.

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